TITLE OF THE INVENTION

REGENERATION AND GENETIC TRANSFORMATION OF *ACACIA MANGIUM*

BACKGROUND OF THE INVENTION

Forests are very important to the world economy and for maintaining and preserving our ecosystem. Forest trees have a wide range of commercial uses (timber for construction, raw material for paper and pulp production, and as an energy source). The global demand for wood products (mostly for paper and pulp and firewood in developing countries) has been increasing year by year when the natural forests are in short supply. Re-forestation is the solution to meeting such increasing demand. Usually, the fast-growing, widely adapted tree species are chosen for re-forestation. Most tree-improvement programs are based on the management of genetic resources, including the selection of superior clones from existing forests, the conservation of genetic variability, partially controlled propagation and classical breeding for desired traits. In spite of the fact that it usually takes several generations to breed, this traditional breeding has been successful in getting elite trees with fast and uniform growth. However, many other traits such as disease and insect resistance, different lignin composition and content are difficult to acquire mainly due to high heterozygosity in tree species and big segregation population. Moreover, the gene(s) conferring certain phenotypes like disease resistance may not be in the gene pool at all. On the other hand, molecular breeding based on genetic transformation of tree species offers the possibility to introduce a particular phenotype without affecting the genetic background of a cultivar. Genetic transformation in Populus species and Eucalyptus species enabled some success in modification of lignin content (Tzfira et al., 1998; Robinson, 1999). The precondition of molecular breeding of forest tree species is the availability of a reliable and reproducible genetic transformation method, which in turn relies on a system of regeneration of one whole plant from a single cell.

Genus *Acacia* comprises about 1200 tropical and subtropical tree species. It belongs to the family *Mimosaceae*. *Acacia mangium* is a multipurpose, fast growing and nitrogen fixing elite tropical legume tree. An adult tree is up to 30-metres tall and its bole (trunk) is often straight to over half the total height. The true leaf of a seedling is a fern-like pinnate leaf. The first pinnate leaf of a seedling has 6 or 8 leaflets, and then the bipinnate leaf develops from the second pinnate leaf on a seedling. Usually when the young seedling grows up to 8-12 bipinnate leaves, the petiole is dilated into a phyllode, while the leaflets abort completely and the true leaf disappears

from the young seedling. Phyllodes are flattened leaf stems which look and act like the ordinary leaves of other plants. Branchlets, phyllodes and petioles are glabrous or slightly scurfy. Phyllodes are 5-10 cm broad, 2-4 times as long as broad, dark green, and are chartaceous when dry. The phyllodes have 3-4 longitudinal main nerves which join on the dorsal margin at the base of the phyllode. Secondary nerves are fine and inconspicuous.

Flowers are in loose spikes to 10 cm long and are solitary or paired in the upper axils. Flowers are pentamerous and the calyx is 0.6-0.8 mm long with short obtuse lobes. The corolla is twice as long as the calyx. Pods are linear, glabrous, 3-5 mm broad, about 7.5 cm long when green, woody, coiled and brackish-brown when mature, and depressed between the seeds. Seeds are lustrous, black, ellipsoid, ovate or oblong, 3.5×2.5 mm, with the orangish funicle forming a fleshy aril beneath the seed (Duke, 1984). Due to *A. mangium* fast growth, tolerance of infertile soil and high quality fibre, it has been increasingly used for reforestation plantation and soil rehabilitation in degraded soil. Its plantation has been studied for many years in many countries or regions, especially in tropical regions and subtropical regions, such as Australian, Indonesia, Malaysia, India, Thailand, Hawaii, China and Taiwan. Many *A. mangium* plantations have been established in acidic soil or abandoned land or Imperata grassland, for example, in Bangladesh (Latif et al., 1995); in Sabah (Latif et al., 1995; Williams et al., 1992) and in Serdong (Majid et al., 1994; Awang, 1994) in Malaysia; Sangmelina, Cameroon, Kenya (Duguma et al., 1994); Skaerat, Thailand (Khemnark, 1994); Hawaii, USA (Cole et al., 1996); Bogor (Anwar, 1992; Wibowo et al., 1992), Paseh and Kadipaten (Widiarti and Alrasjid, 1987) in Indonesia; Bengal, India (Basu et al., 1987), etc.

Indonesia, with some of the world's largest paper and pulp mills, has been increasingly relying on plantations as the source of wood and *A. mangium* is the preferred choice. Asia Paper and Pulp group has two affiliate companies with a total concession of 540,000 hectares. By 1996, one company had planted 123,000 hectares of *A. mangium*, about 90% of all its plantation, which represented 180 million seedlings. It is estimated that by 2004, Asia Paper and Pulp group will virtually source all its wood from plantation, mainly *A. mangium* plantation (Bayliss, 1998a; Bayliss, 1998b).

Besides its use for paper and pulp, *A. mangium* timber can be used for other applications such as cement banded particleboard, plywood and decorative panel manufacture (Yusoff et al., 1989; Wong et al., 1988).

A large scale of mono-plantation of vegetative propagation from a single plant faces a higher risk of disease infection. It has been found that diseases spread very fast in this kind of plantation and cause large economic losses. Many diseases devastate *A. mangium*: Cinnamon fungus (*Phytophehera cinnamomi)* infection results in collapse and death of plants; Seedling blight, defoliation and dieback due to *Glomerella cingulata* caused serious losses to *A. mangium* in nurseries. Leaf spot by *Cyclindrocladium quinqueseptatum* causes defoliation of seedlings and young trees; Powdery mildew (*Oidium spp*) severely affected *A. mangium* seedlings in nurseries in Thailand; Red rot diseases, caused by *ganoderma sp.* affects *A. mangium* in Malaysia; Brown root disease caused by *Phellinus noxium* affects *A. mangium* in Malaysia and the Solomon islands (Simmons, 1987; Gutteridge and Shelton, 1994). Traditional breeding has been less successful in getting disease resistance in *A. mangium*, mostly likely due to lack of such a gene in the natural gene pool. Molecular breeding by introducing foreign disease resistance genes becomes an important option.

Studies on *A. mangium* tissue culture have been limited to micropropagation (Bhaskan and Subbash, 1996; Ahmad, 1991; Galiana et al., 1991a; Galiana et al., 1991b). A combination of traditional breeding of elite trees and techniques of large-scale vegetative propagation make the large-scale plantation possible. There has been no report on regeneration or genetic transformation of *A. mangium*. Our invention describes the conditions for regeneration of whole plant via organogenesis and a genetic transformation system.

The publications and other materials used herein to illuminate the background of the invention or provide additional details respecting the practice, are incorporated by reference, and for convenience are respectively grouped in the appended List of References.

SUMMARY OF THE INVENTION

A. System of regeneration from callus in *Acacia mangium*

Different parts of *Acacia mangium* seedlings or vegetative micropropagated plantlets (hypocotyl, cotyledon, leaflet, petiole and stem) were used as explants. The seedlings were from isolated seed embryo culture and the plantlets were obtained by micropropagation from meristem regions of a 2-year old tree. Callus formation was induced by MS basic medium (Murashige and Skoog, 1962) supplemented with auxins (either 2,4-D from 0.5 mg/L to 5 mg/L or α-

naphthaleneacetic acid (NAA) from 0.5 mg/L to 2 mg/L) and cytokinins (either kinetin (KT) 0.5 mg/L to 3.0 mg/L or 6-benzylaminopurine (6-BA or BA) 0.5 mg/L to 3.0 mg/L).

Adventitious bud induction was achieved on medium AM-265 (MS basic medium with thidiazuron (1-phenyl-3-(1,2,3-thiadiazol-5-yl)urea or TDZ) 1.0 mg/L, indole-3-acetic acid (IAA) 0.25 mg/L, casein enzymatic hydrolysate (CH) 100 mg/L, L-ascorbic acid (vitamin C or Vc) 100 mg/L, L-glutamine (Gln) 150 mg/L, L-asparagine monohydrate (Asn) 150 mg/L, L-proline (Pro) 150 mg/L, pH 5.8 after autoclaving, phytagel 0.275 or 0.30%, or agar 0.8%, sucrose 30 g/L) under the growth conditions of 16/8 hours light/dark (L/D) cycle and at 28°C.

Pinnate leaf formation and adventitious bud elongation were obtained on medium AM-337 (MS basic medium with TDZ 0.01 mg/L, CH 100 mg/L, Vc 100 mg/L, Gln 150 mg/L, Asn 150 mg/L and Pro 150 mg/L, pH 5.8 after autoclaving, phytagel 0.275 or 0.30%, or agar 0.8%, sucrose 30 g/L), or AM-41 (MS basic medium supplemented with 6-BA 2 mg/L, CH 100 mg/L, Vc 100 mg/L, Gln 150 mg/L, Asn 150 mg/L and Pro 150 mg/L, pH 5.8 after autoclaving, phytagel 0.275 or 0.30%, or agar 0.8%, sucrose 30 g/L) supplemented with gibberellic acid (GA_3) 0.5-2.5 mg/L. The growth conditions are 16/8 hrs (L/D) cycle and at 28°C.

Adventitious buds formed roots and complete plantlets were obtained on MS or ½MS basic medium supplemented with NAA 0.5 to 3.0 mg/L combined with KT 0-0.5 mg/L or IBA (indole butyric acid) 0.01-0.5 mg/L and CH 100 mg/L, Vc 100 mg/L, Gln 150 mg/L, Asn 150 mg/L and Pro 150 mg/L, pH 5.8 after autoclaving, phytagel 0.35%, or agar 1.4%, sucrose 20 or 30 g/L under the growth conditions of 16/8 hrs (L/D) cycle and at 28°C. Complete plantlets were transplanted into peat soil:white sand (3:1) in a growth chamber at a photoperiod of 16/8 hrs (L/D) and 25°C.

B. System of genetic transformation of *A. mangium*

Based on the above established regeneration system, Agrobacterium strain LBA4404 (Ooms et al., 1981) harbouring binary vector pBI121 in which a GUS gene is under regulation of an 800 bp cauliflower mosaic virus promoter was used for genetic transformation of *A. mangium*. The Agrobacteria with the plasmid were co-cultured with the following explants: (1) Young adventitious shoots vegetatively micropropagated from auxiliary buds dissected from a 2-year old tree. The shoots consisted of young stem and pinnate leaves but no phyllodes. Stem pieces of about 2-3 cm in length after separating pinnate leaf and auxiliary buds were used as

explants. (2) Petiole pieces of 1-2 cm in length and leaflet pieces of 0.4-0.5×0.3-0.4 (cm) from seedlings derived from embryo culture were used as explants. The explants were pre-cultured on AM-265 for three days at 16/8 hrs (L/D) cycle and at 28°C before infection with activated Agrobacterium for 15 minutes. Infected explants were then cultured for 3 days in the same media in the dark at 22°C. They were then washed and put into AM-265 to induce callus and adventitious buds. Transgenic plants were selected with G418 in media in four stages: G418 12 mg/L for 25 days, G418 20 mg/L for 60 days, G418 30 mg/L for 25 days and then on G418 12 mg/L. Adventitious bud elongation was induced by AM-265 with GA_3 2.5 to 5 mg/L in the presence of G418 12 mg/L. For pinnate leaf formation, adventitious buds were transferred into AM-41 with GA_3 2.5 mg/L at growth conditions of 16/8 hours (L/D) cycle and 28°C. Regenerated shoots were excised and checked for GUS staining and 16% tested positive. Putative transgenic adventitious buds were transferred into AM-357 or AM-451 with or without G418 (10 mg/L) at the photoperiod of 16/8 hours (L/D) to induce rooting. Southern blot hybridization showed positive results when NPTII gene was used as probe.

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BRIEF DESCRIPTION OF THE FIGURES

Figures 1A-E show adventitious bud induction from leaf. Figure 1A shows callus induction. Figure 1B shows adventitious bud recovery. Figures 1C, 1D and 1E show plumule formation and adventitious bud elongation.

Figures 2A-C show *Acacia mangium* regeneration from petiole. Figure 2C shows callus induction. Figure 2B shows adventitious bud recovery. Figure 2C shows plumule formation and adventitious bud elongation.

Figures 3A-E demonstrate *Acacia mangium* regeneration from stem pieces. Figure 3A shows regeneration from callus. Figure 3B shows adventitious buds induced from callus. Figure 3C show root formation, Figure 3D shows a complete plantlet and Figure 3E shows a one month old plantlet in pot soil.

Figure 4 is a map of pBI121 with an 800 bp HindIII-BamHI fragment containing the cauliflower mosaic virus (CaMV) 35S promoter cloned upstream of the GUS gene.

Figures 5A-C demonstrate rejuvenation of a tree. Figure 5A shows a two-year old tree. Figure 5B shows adventitious bud induction. Figure 5C shows propagated adventitious buds with plumules.

Figure 6A shows adventitious buds and Figure 6B shows stem pieces as explants for transformation.

Figure 7 demonstrates selection and induction of putative transgenic adventitious buds.

Figures 8A and 8B illustrate GUS staining of adventitious buds after selection for 5 months.

Figures 9A-C illustrate GUS staining of young transgenic stem pieces. Figures 9A-B show stem pieces and Figure 9C shows a shoot.

Figures 10A-C show GUS staining of transgenic leaf and leaf pieces.

Figure 11 shows the results of a Southern blot to a NPTII probe. 20 μg of DNA was digested with HindIII, run on a gel and blotted. Lanes 1-6 are transgenic lines. Lane ck is a negative control using DNA from a non-transgenic plant. Lane 7 is a positive control using DNA from a tomato transgenic line by plasmid pWS42 with NPTII as the selection marker.

DETAILED DESCRIPTION OF THE INVENTION

The present invention is directed to methods for genetically transforming tissue of trees, especially *Acacia mangium*, and regenerating whole plants via organogenesis from the transformed tissue.

The present invention is further detailed in the following Examples, which are offered by way of illustration and are not intended to limit the invention in any manner. Standard techniques well known in the art or the techniques specifically described below are utilized.

EXAMPLE 1

Reagents and Culture Media

A. MS basic medium: MS medium (Murashige and Skoog, 1962)

Name	Molecular formula	Concentration (mg/L)	Sigma Cat. No.
Macronutrients			
Ammonium Nitrate	NH_4NO_3	1,650	A-3795
Potassium Nitrate	KNO_3	1,900	P-8291
Calcium Chloride dihydrate	$CaCl_2.2H_2O$	440	C-2536
Magnesium Sulfate Heptahydrate	$MgSO_4.7H_2O$	370	M-7774
Potassium phosphate monobasic, anhydrous	KH_2PO_4	170	P-8416
Ferrous Sulfate Heptahydrate	$FeSO_4.7H_2O$	27.8	F-8263
Ethylenediamine-tetraacetic acid (EDTA)	$C_{10}H_{14}N_2O_8Na_2.2H_2O$ (Na_2EDTA)	37.3	E-6635

Micronutrients			
Potassium Iodide	KI	0.83	P-8166
Boric Acid	H_3BO_3	6.2	B-9645
Manganese Sulfate monohydrate	$MnSO_4.H_2O$	16.9	M-7899
Zinc Sulfate	$ZnSO_4.7H_2O$	8.6	Z-1001
Molybdic Acid (Sodium salt:dihydrate)	$Na_2MoO_4.2H_2O$	0.25	M-1651
Cupric Sulfate (Pentahydrate)	$CuSO_4.5H_2O$	0.025	C-3036
Cobalt Chloride (hexahydrate)	$CoCl_2.6H_2O$	0.025	C-2911
Organic Reagents			
myo-Inositol	$C_6H_{12}O_6$	100	I-3011
Nicotinic acid	$C_6H_5NO_2$	0.5	N-0765
Glycine	$C_2H_5NO_2$	2.0	G-6143
Thiamine (Vitamin B1)	$C_{12}H_{17}ClN_4OS.HCl$	0.1	T-3902
Pyridoxine (Vitamin B6) Hydrochloride	$C_8H_{11}NO_3.HCl$	0.5	P-9755

B. Other Organic Reagents

Name	Molecular formula	Sigma Cat. No.
L-Ascorbic acid (Vitamin C)	$C_6H_8O_6$	A-2174
Casein Enzymatic Hydrolysate (CH)		C-7290
L-Glutamine (Gln)	$C_5H_{10}N_2O_3$	G-9273
L-Asparagine monohydrate (Asn)	$C_4H_8N_2O_3.H_2O$	A-4284
L-Proline (Pro)	$C_5H_9NO_2$	P-4655
Sucrose		S-5390

C. Plant Growth Regulators

Name	Sigma Cat. No.
Indole-3-acetic Acid (IAA)	I-2886
α-Naphthaleneacetic Acid(NAA)	N-0640
1-Phenyl-3-(1,2,3-thiadiazol-5-yl)Urea (Thidiazuron, TDZ)	P-6186
6-Benzylaminopurine (6-BA)	B-3408
Kinetin (KT)	K-0753
Gibberellic Acid (GA_3)	G-7645

D. Agar

Name	Sigma Cat. No.
Type M	A-4800
Purified	A-7921
Phytagel	P-8169

E. Antibiotics.

Timentin (T)	Beecham Pharmaceuticals (Pte) Ltd
Kanamycin (K)	Sigma
Genetins (G418 Sulfate)	Clontech 8056-2

F. Media for callus induction

AM-5	MS + 2,4-D 2.0 mg/L + KT 3.0 mg/L
AM-6	MS + 2,4-D 2.0 mg/L + KT 0.5 mg/L
AM-7	MS + NAA 2.0 mg/L + KT 3.0 mg/L
AM-8	MS + NAA 2.0 mg/L + KT 0.5 mg/L
AM-14	MS + 2,4-D 0.5 mg/L + 6-BA 3.0 mg/L
AM-15	MS + 2,4-D 1.0 mg/L + 6-BA 3.0 mg/L
AM-16	MS + 2,4-D 2.0 mg/L + 6-BA 3.0 mg/L
AM-17	MS + 2,4-D 0.5 mg/L + 6-BA 0.5 mg/L
AM-18	MS + 2,4-D 1.0 mg/L + 6-BA 0.5 mg/L
AM-19	MS + 2,4-D 2.0 mg/L + 6-BA 0.5 mg/L
AM-20	MS + 2,4-D 0.5 mg/L + 6-BA 1.0 mg/L
AM-21	MS + 2,4-D 1.0 mg/L + 6-BA 1.0 mg/L
AM-22	MS + 2,4-D 2.0 mg/L + 6-BA 1.0 mg/L
AM-27	MS + NAA 0.5 mg/L + 6-BA 3.0 mg/L
AM-28	MS + NAA 1.0 mg/L + 6-BA 3.0 mg/L
AM-29	MS + NAA 2.0 mg/L + 6-BA 3.0 mg/L
AM-30	MS + NAA 0.5 mg/L + 6-BA 0.5 mg/L
AM-31	MS + NAA 1.0 mg/L + 6-BA 0.5 mg/L
AM-32	MS + NAA 2.0 mg/L + 6-BA 0.5 mg/L
AM-33	MS + NAA 0.5 mg/L + 6-BA 1.0 mg/L
AM-34	MS + NAA 1.0 mg/L + 6-BA 1.0 mg/L
AM-35	MS + NAA 2.0 mg/L + 6-BA 1.0 mg/L
AM-231	MS + 2,4-D 5.0 mg/L + KT 0.5 mg/L
AM-233	MS + 2,4-D 0.5 mg/L + KT 1.0 mg/L
AM-234	MS + 2,4-D 1.0 mg/L + KT 1.0 mg/L

G. Media for adventitious buds induction

a. AM-265: MS basic medium with TDZ 1.0 mg/L, IAA 0.25 mg/L, CH 100 mg/L, Vc 100 mg/L, Gln 150 mg/L, Asn 150 mg/L and Pro 150 mg/L, pH 5.8 after autoclaving, phytagel 0.275 or 0.30%, or agar 0.8% (A-4800, Sigma), sucrose 30 g/L.

b. AM-261: MS basic medium with TDZ 1.0 mg/L, IAA 0.5 mg/L, CH 100 mg/L, Vc 100 mg/L, Gln 150 mg/L, Asn 150 mg/L, Pro 150 mg/L, pH 5.8 after autoclave, phytagel 0.275 or 0.30%, or agar 0.8% (A-4800, Sigma), sucrose 30 g/L.

c. AM-304: MS basic medium with TDZ 2.0 mg/L, IAA 0.25 mg/L, CH 100 mg/L, Vc 100 mg/L, Gln 150 mg/L, Asn 150 mg/L and Pro 150 mg/L, pH 5.8 after autoclave, phytagel 0.275 or 0.30%, or agar 0.8% (A-4800, Sigma), sucrose 30 g/L.

d. AM-308: MS basic medium with TDZ 1.0 mg/L, IAA 2.0 mg/L, CH 100 mg/L, Vc 100 mg/L, Gln 150 mg/L, Asn 150 mg/L and Pro 150 mg/L, pH 5.8 after autoclaving, phytagel 0.275 or 0.30%, or agar 0.8% (A-4800, Sigma), sucrose 30 g/L.

H. Media for pinnate leaf formation

a. AM-337: MS basic medium with TDZ 0.01 mg/L, CH 100 mg/L, Vc 100 mg/L, Gln 150 mg/L, Asn 150 mg/L and Pro 150 mg/L, pH 5.8 after autoclaving, phytagel 0.275 or 0.30%, or agar 0.8% (A-4800, Sigma), sucrose 30 g/L.

b. AM-41: MS basic medium supplemented with 6-BA 2 mg/L, CH 100 mg/L, Vc 100 mg/L, Gln 150 mg/L, Asn 150 mg/L and Pro 150 mg/L, pH 5.8 after autoclaving, phytagel 0.275 or 0.30%, or agar 0.8%, sucrose 30 g/L.

I. Media for root formation

a. Modified AM-8: MS basic medium with NAA 2.0 mg/L, KT 0.5 mg/L, CH 100 mg/L, Vc 100 mg/L, Gln 150 mg/L, Asn 150 mg/L and Pro 150 mg/L, pH 5.8 after autoclaving, phytagel 0.30%, sucrose 30 g/L.

b. AM-357: 1/2 MS basic medium with NAA 2.0 mg/L, KT 0.5 mg/L, CH 100 mg/L, Vc 100 mg/L, Gln 150 mg/L, Asn 150 mg/L and Pro 150 mg/L, pH 5.8 after autoclaving, phytagel 0.30%, sucrose 30 g/L.

c. AM-451: 1/2 MS basic medium with NAA 2.0 mg/L, KT 0.1 mg/L, CH 100 mg/L, Vc 100 mg/L, Gln 150 mg/L, Asn 150 mg/L and Pro 150 mg/L, pH 5.8 after autoclaving, phytagel 0.35%, sucrose 20 g/L.

J. Transplantation

Peat soil:white sand 3:1

K. Media for *Agrobacterium tumefaciens* activation

LB medium

Tryptone	1.0%
Yeast extract	0.5%
NaCl	0.8%

pH 7.0 before autoclaving (Sambrook et al.,1989)

YEP medium (Per liter) (Chilton et al., 1974)

Bactopeptone	10 g
Yeast extract	10 g
NaCl	5 g

AB medium

20 x Phosphate Buffer (per liter)

K_2HPO_4	60 g
NaH_2PO_4	20 g

Autoclave this solution separately

20 x Salts solution (per liter)

NH_4Cl	20 g
$MgSO_4.7H_2O$	6 g
KCl	3 g
$CaCl_2$	0.2 g
$FeSO_4.7H_2O$	0.05 g

pH to 7.0 before autoclaving

To make up the final medium, combine (to a final volume of 1 liter):

50 mL 20 x Phosphate Buffer

50 mL 20 x Salts Solution

900 mL sterile ddH_20

Induction medium

MES buffer, pH 6.0	30 mM
1×AB medium	
Glucose	0.5%
Acetosyringone	100 µM (a stock solution of acetosyringone must be made up fresh in DMSO)

Example 2

Assay for GUS Activity

Stain the tissue overnight at 37°C in GUS staining solution. Gus staining solution is described by Jefferson (1987). It is X-gluc - 1 mM, sodium phosphate (pH 7.0) - 100 mM, EDTA - 10 mM, and Triton X-100 - 0.1%. GUS staining showed a positive blue reaction in adventitious buds and stem and leaf (Figures 8, 9, and 10).

Example 3

Southern Analysis

A method for performing Southern blots is described in Sambrook et al. (1989). The method is:

A. DNA extraction

2-5 g fresh sample was frozen in liquid N_2. This was ground with a mortar and pestle in liquid nitrogen to a fine powder. The powder was transferred to a centrifuge tube (50 mL). 15 mL of Extraction Buffer was added, 2 mL 10% SDS was added and mixed thoroughly. This was incubated at 65°C for 15 minutes. 5 mL 5 M KAc was added and shaken vigorously. The mixture was incubated in ice for 20 minutes and then spun at 25,000 x g for 20 minutes. The supernatant was filtered through Microcloth into a new tube. The DNA was precipitated with ½ volume of isopropanol, mixed and incubated at -20°C for 30 minutes.

The DNA was pelleted at 25,000 x g for 30 minutes, the supernatant was poured off, and the tube was inverted and air dried for 30 minutes. The pellet was dissolved with 0.7 mL of 1×TE (pH 8.0) and transferred to an Eppendorf tube. This was spun 10 minutes. The supernatant was transferred to a new tube, 7 µL RNase (10 mg/mL) was added and left at room temperature for 10 minutes, then 75 µL 3 M NaAc (pH 5.3) and 500 µL of isopropanol were added. The solution was mixed and the DNA was pelleted in a microcentrifuge at full speed for 5 minutes. The pellet was washed with 70% ethanol, air dried and dissolved with 100 µL 1×TE (pH 8.0).

B. Extraction buffer

100 mM Tris-HCl, pH 8	50 mL
50 mM EDTA, pH 8	50 mL
500 mM NaCl	50 mL
10 mM β-ME	0.6 mL
ddH_2O	to 500 mL

C. Enzyme digestion

Reaction system I: DNA sample 100 μL (20 μg), 10× HindIII buffer 40 μL, HindIII 8 μL (80 units), add sterile double distilled H_2O 252 μL to total volume 400 μL.

The reaction was incubated at 37°C for overnight. 40 μL of 3 M NaAc (pH 5.3) and 2/3 volume of 100% ethanol were added to the reaction system and this was incubated at -20°C for 30 minutes. This was spun at full speed at 4°C for 20 minutes. The supernatant was poured off and the tube was air dried for 30 minutes then the pellet was dissolved in 30 μL sterile double distilled water.

D. Electrophoresis

Electrophoresis was performed on a 0.8% agarose gel in 1×TBE at 28V for overnight.

E. Transfer of DNA from Agarose gels to Nylon Membranes

This step was performed as described in Sambrook et al. (1989).

F. Prehybridization

A prehybridization solution of 6×SSC, 5×Denhardt's reagent, 0.5% SDS, 100 μg/mL denatured, fragmented salmon sperm DNA (Stratagene products) and 50% formamide was prepared. 50×Denhardt's is: 5 g of Ficoll, 5 g of polyvinylpyrrolidone, 5 g of bovine serum albumin and ddH_2O to 500 mL, filtered and stored at -20°C.

After fixing DNA to the membrane, the membrane was placed into a hybridization tube containing suitable prehybridization solution using 0.2 mL prehybridization solution for each square centimeter of nylon membrane. The membrane was incubated at 42°C for 6 hours.

G. Probe label

During the prehybridization, labeled probe was prepared using a Boehringer Mannheim-High Primer DNA Labeling Kit. 50 ng of NPTII were added then brought to a volume of 8 µL with H_2O. The DNA was denatured in a 100°C heat block for 10 minutes, chilled quickly in ice, and pulse spun.

On ice the denatured DNA was mixed with: 4 µL High Prime reaction mixture, 3 µL of dATP, dGTP, dTTP mixture, and 5 µL of α-^{32}P dCTP, 3000 Ci/mmol (Biolab). This was incubated at 37°C for 10 minutes. The reaction was stopped by adding 20 µL of 50 mM EDTA (pH 8.0).

The labeled probe was purified by running through a small Sephadex G50 column prepared on a small Pasteur pipette. The eluent was monitored with a counter and the first peak was collected.

H. Hybridization

The probe was added into the hybridization tube, then incubated at 42°C for 10-24 hours.

I. Washing membrane

The hybridization solution was poured off and the membrane was submerged in 2×SSC, 0.5% SDS at room temperature for 10 minutes. The membrane was transferred into 2×SSC, 0.1% SDS at room temperature for 15 minutes. The solution was replaced with 0.5×SSC, 0.1% SDS and the membrane was incubated at room temperature for 15 minutes. The solution was replaced with 0.1×SSC, 0.1% SDS and incubation continued at 55°C for 30-55 minutes. The membrane was transferred into 0.1×SSC at room temperature for 3-5 minutes, then air dried on 3MM Whatman paper for 30 minutes.

J. Autoradiography

The membrane was exposed to X-ray film (Kodak) to obtain an autoradiographic image at -80°C for one day or more.

Using NPTII fragment as a probe, Southern blotting showed that the NPTII gene had integrated in adventitious buds (Figure 11). The results demonstrate that this protocol of *Acacia mangium* transformation is very successful.

EXAMPLE 4

Regeneration of *A. mangium*

A. Embryo culture

Mature seeds (black coat) were pre-treated with 98% H_2SO_4 for 2-3 minutes and washed with tap water several times. Treated seeds were sterilized with 70% ethanol for 2-3 minutes and washed 5 times with sterile ddH_2O. Seeds were then immersed in 0.1% $HgCl_2$ for 6 minutes and washed 5 times with sterile ddH_2O, again sterilized with bleach 30% (market product) for 6 minutes, then washed 5 times with sterile ddH_2O. Sterilized seeds were soaked in sterile ddH_2O overnight for isolating zygotes for embryo culture. MS basic medium (Murashige and Skoog, 1962), pH 5.8, sucrose 30 g/L, phytagel 0.25% or agar 0.7% (Sigma, A-4800), with or without activated charcoal, was used to culture mature embryo. Isolated mature zygotic embryos were cultured on MS using a photoperiod of 12/12 hours or 16/8 hours (L/D) or complete dark, at 25-28°C. Germinated hypocotyls or leaves or petioles or stems were used as explants to induce callus formation.

B. Callus induction

Different media were used to induce callus, including medium Nos. AM-5, 6, 7, 8, 14, 15, 16, 17, 18, 19, 20, 21, 22, 27, 28, 29, 30, 31, 32, 33, 34, 35, 231, 233 and 234 at 12/12 hours or 16/8 hours (L/D) or Dark at 25°C. Callus induced from young leaflet: AM-L; from young petiole: AM-P; from hypocotyl: AM-H; from young stem: AM-S; from bud: AM-B; from root: AM-R. All above calli were used to induce adventitious buds, using a photoperiod of 16/8 hours, 1800-2000 lux, and 28°C. The results of callus induction on different media were different (Table.1). Generally callus induction was not difficult. All explants produced calli at the photoperiod of 12/12 hrs or 16/8 hrs (L/D) (see, e.g., Figure 1A; Figure 2A; Figure 3A), and 25°C for 20 days.

Table 1

Results of callus induction on different media

at 12/12 hours (L/D), 25°C cultured for 35 days

Medium No.	Number of Explants (leaflet)	No. of explants forming callus	No. of explants complete callus formation
AM-5	40	40	33
AM-6	40	40	31
AM-7	40	40	3
AM-8	40	40	4
AM-14	40	40	36
AM-15	40	40	40
AM-16	40	40	38
AM-17	40	40	36
AM-18	40	40	39
AM-19	40	40	39
AM-20	40	40	38
AM-21	40	40	40
AM-22	40	40	40
AM-27	40	40	12
AM-28	40	40	8
AM-29	40	40	11
AM-30	40	40	10
AM-31	40	40	6
AM-32	40	40	2
AM-33	40	40	4
AM-34	40	40	4
AM-35	40	40	6
AM-231	40	40	30
AM-233	40	40	20
AM-234	40	40	20
	Petiole		
AM-6	20	20	15
AM-17	20	20	20

C. Adventitious bud induction

Calli induced from leaflet, petiole, young stem and buds were cultured on AM-261, AM-265, AM-304, and AM-308, at a photoperiod of 16/8 hrs (L/D), at 28°C. One month later, there were some bud recoveries from callus (Figure 1B; Figure 2B). Bud recovery from friable callus first appeared as compact and smooth bud terminal. The ratio of bud recovery reaches 15% on AM-265. But on these media, induced buds could not easily form pinnate leaves and elongate.

D. Pinnate leaf formation and adventitious bud elongation

Bud recoveries from callus were transferred into AM-337 or AM-41 with GA_3 2.5 mg/L, at a photoperiod of 16/8 hrs (L/D), 1800-2000 lux, and 28°C. After culture for one month, some of them could form pinnate leaves and elongate (Figures 1C, D, E; Figure 2C; and Figure 3B). The ratio of pinnate leaf formation and bud elongation reaches 16.67%.

E. Root formation

Elongated buds were cultured in AM-357 or AM-451 for root formation. 20 days later, adventitious roots began to appear from basic stem of buds (Figure 3C). After root formation, plantlets were transferred to MS basic medium without any plant growth regulators. Plantlets grow normally and fast, and the root system grew well (Figure 3D).

F. Transplantation

Phytagel on plantlets was washed away and the plantlets were transferred to pot (peat soil:white sand 3:1) and grown in a growth chamber at a photoperiod of 16/8 hours and 25°C. One month later the plantlets were transferred to a green house (Figure 3E).

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Example 5

Rejuvenation of Tree

The auxiliary buds of a two-year old young tree (Figure 5A) were cultured on MS basic medium with NAA 0.1 mg/L, 6-BA 3.0 mg/L, CH 100 mg/L, Vc 100 mg/L, Pro 150 mg/L, Asn 150 mg/L and Gln 150 mg/L, phytagel 0.275% or agar 0.8% (A-4800, Sigma), pH 5.8 after autoclaving at 121°C, sucrose 30 g/L. Sixty days later, some adventitious buds with phyllodes were obtained (Figure 5B). Induced adventitious buds with phyllodes were subcultured on AM-41 (MS basic medium with 6-BA 2 mg/L, CH 100 mg/L, Vc 100 mg/L, Pro 150 mg/L, Asn 150 mg/L and Gln 150 mg/L, phytagel 0.275% or agar 0.8% (A-4800, Sigma), pH 5.8 after autoclaving at 121°C, sucrose 30 g/L). After sub-culturing adventitious buds with phyllodes twice in about two months, the rejuvenation of adventitious shoots with pinnate leaf was obtained (Figure 5C). Adventitious buds can be used as explants for transformation (Figure 6A).

Example 6

A. mangium Genetic Transformation

A. Activation of *Agrobacterium tumefaciens* Strain LBA4404/ pBI121

Agrobacterium strain LBA4404 (Ooms et al., 1981) harbouring binary vector plasmid pBI121 (size of vector 13.0 kb, Clontech, Figure 4) was used for this experiment. pBI121 was derived from pBI101 with an 800-bp HindIII-BamHI fragment containing the cauliflower mosaic virus (CaMV) 35S promoter cloned upstream of the GUS gene. From a permanent glycerol stock stored at -70°C, *Agrobacterium tumefaciens* pBI121/LBA4404 was streaked onto a solid LB (pH 7.0) medium plate containing Streptomycin 100 mg/L (Str^{100}) and Kanamycin 50 mg/L (K^{50}). This was incubated 2-3 days at 28°C. Fresh pBI121/LBA4404 was streaked onto solid LB medium (pH 7.0) with Streptomycin 100 mg/L (Str^{100}) and Kanamycin 50 mg/L (K^{50}) for overnight or 24 hours at 28°C in the dark. Several colonies of pBI121/LBA4404 were picked and pBI121/LBA4404 was inoculated in liquid 50 mL LB medium with Streptomycin 100 mg/L (Str^{100}) and Kanamycin 50 mg/L (K^{50}), and cultured at 28°C under dark, 250 rpm for 10 hours until OD_{600} = 0.70-1.10. This was spun down at 3500 rpm for 30 minutes or 5000 rpm for 10 minutes, and resuspended in 4-5 volumes of YEP medium (pH 7.0) with Streptomycin 100 mg/L (Str^{100}) and Kanamycin 50 mg/L (K^{50}), OD_{600} = 0.10-0.20, incubated at 28°C in the dark, 250 rpm for 8-10 hours, OD_{600} = 0.70-1.20. This was spun down at 3500 rpm for 30 minutes and resuspended in the same volume of sterile NaCl 0.9%. This was spun down at 3500 rpm for 30 minutes and resuspended in 2-3.5 volumes of induction medium, OD_{600} = 0.2-0.3, and incubated at 28°C in the dark, 120 rpm for 8-15 hours, OD_{600} = 0.70-1.20 for infecting explants or callus or a cell suspension.

B. Preculture of stem pieces

Stem pieces were cultured on AM-265 for 3 days using a photoperiod of 16/8 hrs (L/D), 1800-2000 lux, at 28°C.

C. Co-cultivation

After preculture, stem pieces were soaked in 0.5 M mannitol for 20-25 minutes and then transferred to activated pBI121/LBA4404 suspension for 15 minutes. Infected young stem pieces

were washed once and dried on sterile Whatman paper before being cultured on AM-265 with 100 μM acetosyringone at pH 5.2 at 22°C in the dark for 3 days.

D. Selection of transformed adventitious buds and transformed callus

After co-culturing for 3 days, stem pieces were washed with sterile ddH_2O for 10 times and dried on sterile Whatman paper. Then stem pieces were cultured on AM-265 containing Timentin 250 mg/L and G418 12 mg/L, phytagel 0.275%, using a photoperiod of 16/8 hrs (L/D) and 28°C for selecting transformed callus or transformed adventitious buds. Subsequently, stem pieces were cultured on the above media with G418 12 mg/L for 25 days, G418 20 mg/L for 60 days, G418 30 mg/L for 25 days, then on G418 12 mg/L. After continuous selecting for 4 months on antibiotics, 33.75% of stem pieces formed adventitious buds, and 2.5 mg/L to 5 mg/L GA_3 was added into the same media to promote adventitious bud elongation (Figure 7). After selection for five months, Timentin was no longer used in the medium and some adventitious buds were used for GUS staining. GUS staining showed positive blue colour reaction in adventitious buds (Figure 8, Figure 9, and Figure 10). Adventitious buds were transferred to AM-41 with GA_3 2.5 mg/L to promote pinnate leaf formation.

E. Root formation

Transformed adventitious buds were transferred into AM-357 or AM-451 with or without G418 (10 mg/L) using a photoperiod of 16/8 hrs (L/D) at 28°C.

While the invention has been disclosed in this patent application by reference to the details of preferred embodiments of the invention, it is to be understood that the disclosure is intended in an illustrative rather than in a limiting sense, as it is contemplated that modifications will readily occur to those skilled in the art, within the spirit of the invention and the scope of the appended claims.

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